

AD _____

Award Number: DAMD17-99-1-9319

TITLE: TGF-B and Breast Cancer Induction

PRINCIPAL INVESTIGATOR: Branka Brukner Dabovic, Ph.D.

CONTRACTING ORGANIZATION: New York University School of Medicine
New York, New York 10016

REPORT DATE: July 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation

DTIC QUALITY INSPECTED 3

20010110 051

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 074-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503				
1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 2000	3. REPORT TYPE AND DATES COVERED Annual Summary (1 Jul 99 - 30 Jun 00)		
4. TITLE AND SUBTITLE TGF- β and Breast Cancer Induction		5. FUNDING NUMBERS DAMD17-99-1-9319		
6. AUTHOR(S) Branka Brukner Dabovic, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) New York University School of Medicine New York, New York 10016 E-MAIL: dabovb01@med.nyu.edu		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) The breast produces inhibitors of breast tumor formation. We hypothesized increases the amount of these compounds would delay cancer onset. We study the molecule TGF- β which blocks cell growth. TGF- β is produced as latent TGF- β complex consisting of the TGF- β homodimer, the TGF- β propeptide dimer, and a second gene product, the latent TGF- β binding protein (LTBP). LTBP targets latent TGF- β to the extracellular matrix, from which active TGF- β is released. The third cysteine rich repeat (CR3) of LTBP-1 is necessary and sufficient for covalent interaction with small latent TGF- β . CR3 overexpression should result in the TGF- β propeptide complexing to an LTBP form unable to interact with matrix. Therefore, TGF- β in this complex should be more easily activated. We generated mice overexpressing CR3 under the control of breast specific WAP promotor, and will generate mice overexpressing CR3 under the control of MMTV LTR. We will study whether breast cancer occurrence is delayed compared to wt animals. We will test whether tamoxifen treatment, which prevents breast cancer, and overproduction of TGF β in genetically engineered mice block tumorigenesis better than either condition alone.				
14. SUBJECT TERMS Breast Cancer, TGF- β , LTBP			15. NUMBER OF PAGES 12	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

___ Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Bryanne Jabonc' 7/26/2000
PI - Signature Date

Table of Contents

Cover.....	1
SF 298.....	2
Foreword.....	3
Table of Contents.....	4
Introduction.....	5
Body.....	6
Key Research Accomplishments.....	10
References.....	11

Introduction

TGF- β is the most potent inhibitor of the progression of normal mammary epithelial cells through the cell cycle (Robinson et al. 1991). During the early stages of breast cancer development, the transformed epithelial cells are sensitive to TGF- β -mediated growth arrest, and TGF- β acts as an anti-tumor agent. We hypothesized that, if methods were found that increased the amount of naturally produced TGF- β , the onset of cancer might be delayed or prevented. TGF- β is normally produced in an inactive form and must be freed from the inactive complex to be active. The latent TGF- β complex consists of the TGF- β homodimer plus its N-terminal precursor propeptide dimer and is named small latent complex (SLC) (Derynck et al. 1985; McMahon et al. 1996; Miller et al. 1992). The precursor propeptides are disulfide bonded to a second gene product, the latent TGF- β binding protein (LTBP) (Kanazaki et al. 1993), and this complex is called the large latent complex (LLC). Most cells (such as T47-D breast cell carcinoma cells) produce TGF- β as part of the LLC (Miyazono et al. 1993; Sporn et al. 1992; Masague et al. 1992; Harpel, 1998). LTBP targets latent TGF- β to the extracellular matrix, from which biologically active TGF- β may be released. Our lab demonstrated that the third cysteine rich repeat (CR3) of LTBP-1 is necessary and sufficient for covalent interaction with small latent TGF- β (Gleizes et al. 1996). We reasoned that overexpression of this domain, CR3, should result in all of the TGF- β propeptide complexing to an LTBP1 form that is unable to interact with matrix and therefore would be more easily activated. I proposed to generate genetically engineered mice that express CR3 under the control of breast-specific MMTV and WAP promoters. This should increase the local concentration of TGF β in mammary tissue and suppress mammary tumor formation. As the anti cancer agent tamoxifen is believed to enhance latent TGF- β activation, we also proposed to determine if tamoxifen acted synergistically in delaying tumors in transgene vs. normal mice.

For the first year of this project, we proposed to construct the CR3 transgenes, test the expression of the transgenes in cultured cells, create transgenic mice that express each of two transgenes, and establish lines of mice that express high levels of each transgene. Even though the initiation of the project was delayed because of difficulties obtaining the WAP and MMTV promoter constructs, we have achieved most of our stated goals.

The intent of the proposal was to create a latent complex of TGF- β that was more easily activated than the normal complex. The latent TGF- β complex consists of TGF- β , the TGF- β propeptides, which remain associated with TGF- β by non-covalent interactions, and the latent TGF- β binding protein (LTBP), which is covalently bound to the TGF- β propeptides. Using HT1080 human fibrosarcoma cells as a model for the secretion and proteolytic release of pericellular matrix-associated TGF- β (Taipale et al., 1992; Taipale et al 1994), we have shown that the ECR3E construct, which consists only of the domain that is bound to the TGF- β propeptide plus flanking EGF-like repeats, binds to TGF- β , and that TGF- β in this complex is not incorporated into the matrix (Mazzieri, unpublished results). We have also shown that over expressing the ERC3E results in excess latent TGF- β activation in the skin, and we proposed to characterize its activities when overexpressed in the breast.

Construction of the WAP-ECR3E transgene.

The WAP promoter construct was obtained from a plasmid clone of the coding region for the WAP protein and WAP promoter. This construct was obtained from M. Sternlicht, UCSF. The myc tag on the ECR3E fragment that was suggested in the initial proposal was modified and replaced with hemagglutinin (HA) epitope tag. The reason for this change was the difficulty to detect the myc tag in the skin of K14 - ECR3E myc transgenic animals (Mazzieri, unpublished data). We designed -

Primers CGGGGATCCACTAGTGGATGTGAATGAATGTGAACT and AACAAAGCACT-GCAGTTTCACAG that specifically amplified ECR3E fragment from human LTBP1 cDNA, adding at the same time two HA tags and a stop codon at the the end of ECR3E. The ECR3E-2HA construct was checked after ligation into pBluescript for proper reading frame. The Forward primer was fused to the 3' end of the signal sequence BM40 (SPARC) in the vector pRC/CMV (gift from Rupert Timpl). The ECR3E-2HA fragment was isolated by restriction digestion with the enzymes Asp 718 and BamHI and inserted after the WAP promoter region to replace the WAP coding sequence. The polyadenylation signal was that of the native WAP gene and was contained in the 3' region of the WAP plasmid clone. (Fig. 1). The 4.8 kb fragment obtained after this ligation represented the WAP-ECR3E-2HA construct. The construct was amplified, and the WAP-ECR3E-2HA construct released from the vector by the restriction digestion with EcoRI and purified. The isolated DNA was microinjected into mouse zygotes from the FVB strain. 316 eggs were microinjected with the construct

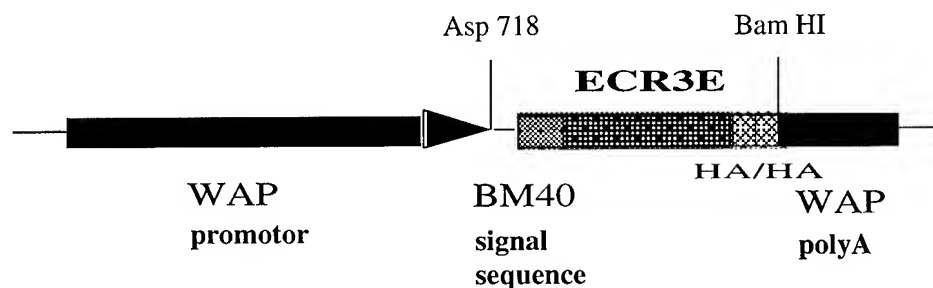


Fig. 1

and 285 were implanted into 9 pseudopregnant females. 42 mice were recovered. The animals were genotyped with specific primers. These span 310 bp corresponding to the region between the 3' end of the WAP promoter (WAP primer: 5' GTAGCCCATCTAGAGCTGTGCC 3') and the 5' end of the ECR3E-2HA fragment (ECR3E primer: 5' CGTTTTACAGAAGGCTTCACC 3'). We have obtained 9 transgenic animals. These animals are now being bred to monitor transmission of the transgene.

The second construct, which we proposed to make, MMTV-ECR3E presented us with several problems. The pMMTV plasmid, containing the complete, hormone-inducible MMTV LTR promoter / enhancer, obtained from H. Moses, Vanderbilt University, did not have convenient restriction sites for the insertion of the ECR3E. An attempt was made to insert the ECR3E fragment using blunt end ligation. However, this failed even though more than 150 bacterial colonies were screened. In all cases in which the ECR3E-2HA fragment had integrated, the orientation was reversed relative to the MMTV promoter. The reason for this failure to obtain integration in the correct orientation is not clear. We have checked many different bacterial strains with the hope that this might overcome the problem, but we have been unsuccessful. We have started two new approaches. In one we are using special adaptors that will insure directional cloning, and in the other we are creating new restriction sites that will insure the correct orientation.

We altered two aspects of our strategy. First, our intention is to mate the transgenic mice we produced with mice that have been genetically modified so that all females get breast tumors. We had proposed to use were the MMTV-pYMT#121 and MT#634 strains (Guy et al., 1992), but the originators of these mice lost them, e.g. the investigators stopped breeding the mice. However, we have found MMTV-neu #202 strain mice that are commercially available (The Jackson Laboratories) and which have similar properties to the animals we proposed to use as that they have 100%

breast cancer penetrance at 140 days. These mice have been obtained, and we will begin to expand their number so that we can cross them with the transgenic animals. Second, we have not checked for the expression of the WAP-ECR3E transgene in cultured cells. Although WAP is the major whey protein expressed in mammary glands in response to lactogenic hormones, little or no WAP expression is detectable in cell culture. (Shoenenberg et al., 1990, Park et al., 1989). Therefore we will check for the expression of WAP-ECR3E in the mammary glands of transgenic females in all WAP-ECR3E mouse lines. We will choose the lines with the highest expression of the transgene for breeding with MMTV-neu #202 mice and further studies on breast cancer formation.

TGF- β and Breast Cancer Induction
Annual Summary Report

Brukner Dabovic, Branka

Key research accomplishments:

- Creation of WAP-ECR3E-2HA transgene.
- Creation of WAP-ECR3E-2HA transgenic mice.

Annual Summary Report

References:

Derynck et al. (1985): Human transforming growth factor- β complementary DNA sequence and expression in normal and transformed cells. *Nature* **316**, 701-705

Gleizes, P-E. Beavis, R.C., Mazzieri, R. Shen, B. and D. Rifkin. (1996): Identification and Characterization of an Eight-cysteine Repeat of the Latent Transforming Growth Factor - β Binding protein that Mediates Bonding to the Latent Transforming Growth Factor- β 1 *J. Biol. Chem.* **271**, 29891-29896.

Guy, C.T., Cardiff, R.D. and W.J. Muller (1992): Induction of Mammary Tumors by expression of Polyomavirus Middle T Oncogene: A Transgenic Mouse Model for Metastatic Disease. *Mol. Cell. Biol.* **12**, 954-961.

Harpe I, J. (1998): Novel proteolytic and non-proteolytic mechanisms for the activation of TGF β . PhD thesis, New York University School of Medicine, New York, USA

Kanzaki, T. et al. (1990): TGF- β binding protein: a component of the large latent complex of TGF- β 1, with multiple repeat sequences. *Cell* **61**, 1051-1061.

Masague J. (1992): Receptors for the TGF- β family. *Cell* **69**, 1067-1070

McMahon, G.A., Dighnam J.D. and L.E. Gentry (1996): Structural characterization of the latent complex between transforming growth factor β 1 and β 1 latency associated peptide. *Biochem J.* **313**, 343-351.

Miller, et al. (1992): Characterization of the binding of transforming factor - β 1, - β 2, and - β 3 to recombinant β 1-latency associated peptide. *Mol Endocrinol.* **6**, 694-702.

Miyazono, K., Ichijo, H. and C.H. Heldin (1993): Transforming growth factor- β latent forms, binding proteins, and receptors. *Growth Factors* **8**, 11-22

Park C.S., Choi Y., Keller W.L. and R.L. Harrold (1989): Effects of compensatory growth on milk protein gene expression and mammary differentiation. *FASEB J.* **2**, 2619-2624
Robinson, S.D., Silberstein, G.B., Roberts, A.B., Flanders, K.C. and C.W. Daniel (1991): Regulated expression and growth inhibitory effect of transforming growth factor isoforms in mouse mammary gland development. *Development* **113**, 867-878.

Annual Summary Report

Shoenenberger, C-A, Zuk, A., Groner B., Jones W. and A-C Andres (1990): Induction of Endogenous Whey Acidic protein (Wap) Gene and a Wap-myc Hybrid Gene in Primary murine mammary Organoids. *Dev. Biol.* **139**, 327-337.

Sporn, M.B. and A.B. Roberts (1991): Transforming growth factor- β : recent progress and new challenges. *J. Cell Biol.* **119**, 1017-1021.

Taipale, J., Koli, K. and J. Keski-Oja (1992): Release of Transforming Growth Factor β 1 from the Pericellular Matrix of Cultured Fibroblasts and Fibrosarcoma Cells by Plasmin and Trombin. *J. Biol. Chem.* **267**, 25378-25384.

Taipale, J., Miyazono, K, Heldin C.H. and J. Keski-Oja (1994): Latent transforming growth factor β 1 associates to fibroblast extracellular matrix via latent TGF- β 1 binding protein. *J. Cell Biol.* **124**, 171-181